Tumor Suppressive Role of an Androgen-regulated Epithelial Cell Adhesion Molecule (C-CAM) in Prostate Carcinoma Cell Revealed by Sense and Antisense Approaches¹

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ABSTRACT

We recently demonstrated that C-CAM, an epithelial-cell adhesion molecule of the immunoglobulin supergene family, could be regulated by androgen and might act as a growth repressor during differentiation of the prostatic epithelium. To define the role of C-CAM in prostatic tumorigenesis, a tumorigenic human prostatic cancer cell line, PC-3, was transfected with an expression plasmid containing C-CAM1 (a C-CAM isoform). Transfected clones showed significantly lower growth rates, reduced anchorage-independent growth, and less tumorigenicity in vivo than control cells. Furthermore, transfection of an antisense vector into a nontumorigenic prostatic epithelial cell line, NbE, resulted in tumor formation in nude mice. Sublines derived from these NbE-induced tumors had lower levels of C-CAM than did control cells. These data suggest that C-CAM1 can function as a tumor suppressor in prostate tumorigenesis.

INTRODUCTION

CAMs³ are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (1). Recent data indicate that aberrant expression of CAMs is involved in the tumorigenesis of many neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (2–6). Also, Giancotti and Ruoslahti (7) demonstrated that increasing expression of αβ₁ integrin by gene transfection can reduce tumorigenicity of Chinese hamster ovary cells in vivo.

We have been studying C-CAM, a CAM predominantly expressed in epithelial cells (8). Molecular cloning of C-CAM cDNA indicates that C-CAM belongs to the carcinoembryonic antigen family and is a member of the immunoglobulin supergene family (9, 10). Thus far, two C-CAM isoforms, C-CAM1 and C-CAM2, have been identified. Both isoforms are composed of a cytoplasmic domain, a transmembrane domain, and an extracellular domain with four immunoglobulin-like domains. C-CAM1 and C-CAM2 differ in their first immunoglobulin domains by 16 amino acids and in the length of their cytoplasmic domains (10). C-CAM1, but not C-CAM2, shows adhesion activity when expressed in a baculoviral vector in insect cells (11). Further structural and functional analyses of C-CAM1 by Cheung et al. (11) indicated that the presence of the first immunoglobulin domain is essential for adhesion. During liver development, expression of both C-CAM isoforms correlates with differentiation of hepatocytes, and the regulation of C-CAM expression is mainly transcriptional (12, 13). These observations suggest that C-CAM, like E-cadherin, plays a role in epithelial cell differentiation and that altered expression of C-CAM may be associated with diseases of epithelial origin. Hixson and McEntire (14) reported an apparent decrease in C-CAM on the cell surface of rat hepatocellular carcinoma lines. In addition, Rosenzweig et al. (15) and Neumaier et al. (16) demonstrated that mouse and human biliary glycoprotein, a C-CAM homologue, is down-regulated in colon tumors. These observations are consistent with the notion that reduction in C-CAM expression may be commonly associated with epithelial malignancy. However, little is known about the underlying mechanism or mechanisms by which CAMs modulate these processes.

The prostate gland is known to be an androgen-dependent organ; that is, glandular epithelium, especially columnar epithelium that has secretory function, undergoes tissue degeneration (i.e., apoptotic death) after androgen deprivation (e.g., by castration). On administration of androgen, the entire degenerated organ returns to its original size with active secretory function and no sign of abnormality, including tissue hyperplasia or malignancy. This process is reversible by repeated cycles of androgen deprivation and androgen supplementation. Therefore, it has been speculated that the enriched stem/amplifying cells remaining in the degenerated organ after castration are preprogrammed to generate only certain numbers of progeny cells, which determine the ultimate size of the prostate (17). Such a highly regulated process may require the presence of putative negative regulatory signals that determine the proliferative potential of those stem/amplifying cells and that alteration of these negative regulators may be an early event in the development of cellular abnormality. Such regulatory signals should be up-regulated in stem/amplifying cells throughout the entire castration period. Most importantly, these negative regulators should inhibit growth. In a recent study (18), we demonstrated that C-CAM was up-regulated in rat prostate after castration and that the elevated C-CAM level was associated with stem/amplifying cells. Also, the level of C-CAM that accumulated in these cells was constant throughout the entire castration period. This phenomenon is not related to apoptosis because the kinetics of C-CAM expression differ from those of apoptosis. As soon as the prostate began to regenerate because of androgen administration, the levels of C-CAM expression diminished from stem/amplifying cells and the basal levels of C-CAM protein reappeared in columnar epithelia. On the basis of these findings, we hypothesized that C-CAM may be a growth repressor in prostate gland development.

Prostate cancer, a disease of epithelial origin, has become the leading cancer among United States men. The mortality associated with this cancer is mostly due to metastatic and recurrent disease. Recurrent prostate cancer cells are rapidly growing, poorly differentiated, and androgen independent. We have observed that C-CAM levels were decreased or diminished in poorly differentiated prostate cancer lines and in patient specimens. Therefore, we decided to examine the function of C-CAM in prostate cancer. In this paper, we demonstrate that expression of C-CAM could suppress the tumorigenicity of human prostate cancer cells. Conversely, we found that

reduced C-CAM expression could lead to tumor formation in a nontumorigenic prostatic epithelial cell line. Thus, C-CAM may play an important role in prostate cancer progression.

MATERIALS AND METHODS

Plasmid Construction and Transfection into PC-3 and NbE Cells. To construct the sense and antisense C-CAM expression plasmids, a cDNA insert (1.7 kilobases) containing the entire coding sequence for C-CAM1 was cloned into the SalI site of the vector pRSN (19), a mammalian expression vector carrying neomycin acetyltransferase gene (neo'), which confers neomycin resistance. The C-CAM1 cDNA is under the control of both the Rous sarcoma virus long terminal repeat and the SV40 early promoter. The orientation of C-CAM1 in pRSN was determined by restriction mapping. To select transfected cells, 5 µg of C-CAM1 expression plasmid mixed in 10 µl of Transfectam (Promega, Madison, WI) was added to parental PC-3 or NbE cells grown in T medium (80% Dulbecco’s MEM 20% Hank’s F-12 medium) containing 5% FBS. To select for neomycin-resistant clones, the transfected cells were grown for 1 month in increasing concentrations of G418. The final concentration of G418 was 800 µg/ml and 1 mg/ml for PC-3 cells and NbE cells, respectively.

Cell Sorting by Flow Cytometry. Membrane fluorescence staining was performed on a single cell suspension by using an anti-C-CAM polyclonal antibody (Ab669) and FITC-conjugated secondary antibodies as described by Cheung et al. (11). FACS was performed with a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) delivering 50 mW at 488 nm with an Enterprise air-cooled laser. The flow cytometer was sterilized by running it for 30 min with 70% ethanol and then equilibrating it with sterile isoton II (Coulter, Hialeah, FL) for 1 h. Log green fluorescence was measured with a bandpass filter (530 nm/30 nm), and triggering mode was executed with forward light scatter set at a sufficiently high threshold to eliminate cell debris and dead cells (<5%). Drop delay was performed using the Autosort procedure (Becton Dickinson, Mountain View, CA). Analysis was performed using LYSYS II software (Becton Dickinson, Mountain View, CA).

Southern, Northern, and Western Blot Analyses. For southern analysis, high-molecular-weight DNA was purified by the procedure of Davis et al. (20). Twenty µg of DNA were digested with restriction endonuclease overnight at 37°C and then subjected to Southern blot analysis as described previously (21) with a C-CAM cDNA probe (10).

For northern analysis, total cellular RNA was extracted from cells by using RNAzol B (Biotex Laboratories, Inc., Houston, TX) and a single-step purification protocol described by Chomczynski and Sacchi (22). Twenty µg of RNA was subjected to northern blot analysis by electrophoresis on a 0.9% agarose gel containing 2 m formaldehyde as described previously (18), and the blot was hybridized with a C-CAM cDNA probe (10).

To isolate total cellular protein for Western analysis, the cells were trypsinized into a single-cell suspension and counted with a hemacytometer. Aliquots of the cell suspension from each clone were boiled in SDS sample buffer, and Western blot analysis was performed as described previously (11) with anti-C-CAM antibody Ab669.

Cell Adhesion Assay. The cells were resuspended at a concentration of 1 X 10⁶/ml. Cell suspensions (1 ml) in 1.5-ml Eppendorf tubes were mixed gently at room temperature to allow formation of aggregates. Samples were taken over a 5-h period, and the number of single cells was determined with a hemacytometer. The aggregation of cells was monitored as a decrease in the percentage of single cells.

In Vitro Growth Assay. The in vitro growth rate of C-CAM1-transfected PC-3 clones was measured with three different assays. In the first method, cells were grown in T medium containing 5% FBS, trypsinized, and counted with a hemacytometer 24, 48, and 96 h after plating. In the second method, 5,000 cells from each clone were grown on a 24-well plate with T medium containing 1% FBS. The medium was changed every 4 days. The number of cells was determined every 48 h by using the crystal violet assay as described previously (23). In the third method, cells were plated similarly; at the times indicated, fresh medium containing 0.1 µCi of [³H]thymidine was added, and the [³H]thymidine incorporation was determined after a 2 h incubation as described previously (24).

Measurement of Anchorage-Independent Growth. The soft agar assay described by Giancotti and Ruoslahti (7) was used to measure the anchorage-independent growth of each transfected clone. Cells were plated onto 6-well plates, and colonies were scored in triplicate by two different investigators 21 days after plating by counting aggregates (>10 cells) in several randomly chosen areas.

Assessment of In Vivo Tumorigenicity. To determine the tumorigenicity of each transfected clone, we injected 1 X 10⁶ cells/site at 6 sites s.c. in the flanks of 8–10-week-old male nude mice. Once a tumor became palpable, it was measured weekly, and its size was calculated using the formula for the volume of a hemiellipsoid, the geometric figure most nearly approximating the shape of the tumor: volume = length x width x height x 0.5236 (25).

In Vitro Cell Cloning of NbE Sublines (Mac). Tumors were excised from animals and placed in serum-free T medium containing antibiotics. After removing excess skin and blood clots, tissues were rinsed with serum-free T medium 3 times. Tissues were cut into 3-mm² pieces and placed in a 6-well plate coated with FBS; then, a small amount of 10% FBS T medium was added to cover each tumor for overnight incubation at 37°C. The medium was changed every 4 days, and G418 (200 µg/ml) was added when epithelia started appearing on the dish. The concentration of G418 was gradually increased to a final concentration of 1 mg/ml within 1 month; the cells were then further cloned by the ring-cloning technique.

RESULTS

Selection of C-CAM1-transfected PC-3 Cells. To examine the role of C-CAM in prostate cancer progression, we transfected the C-CAM1 cDNA in the mammalian expression vector pRSN into a human prostate carcinoma cell line, PC-3. This cell line is highly tumorigenic and negative for C-CAM by both Northern and Western analyses. After transfection, the cells were grown in medium containing G418 to select for neomycin-resistant clones.

Immunofluorescence staining of the transfected PC-3 cells with anti-C-CAM antibody Ab669 was performed after 1 month of G418 selection. Although the immunofluorescence staining showed that C-CAM1 was expressed on the cell surface (Fig. 1C, arrow), only a small portion of cells (about 10%) reacted with the polyclonal antibody against C-CAM (Fig. 1A). This result suggests that only a small proportion of the transfected cells expressed C-CAM1. To increase the proportion of C-CAM1-positive cells for subsequent cloning, the C-CAM1-positive cell population was enriched to 92% by fluorescence-activated cell sorting (Fig. 1B). Two clones, PC-L1 and PC-L2, were isolated from the enriched population and characterized. Two additional clones, PC-L6 and PC-L9, were later isolated from this enriched population using the same approach.

Characterization of C-CAM Integration and Levels in PC-3 Transfectants. Southern blot analysis of genomic DNA isolated from various transfectedants, digested with HindIII, and hybridized with a C-CAM cDNA probe revealed several different DNA integration patterns (Fig. 2A). As expected, the parental PC-3 cells were negative for neo' PC-3 cells transfected with the PC-RSN vector alone (which were called PC-RSN cells) showed a complex pattern of neo' integration. Two patterns of integration were seen among the four C-CAM1-positive clones, PC-L1 and PC-L2 having one type of integration and PC-L6 and PC-L9 having another. These data indicate that these four C-CAM1-positive clones were derived from at least two different cells.

No C-CAM message was detected by Northern blot analysis in PC-3 or PC-RSN cells (Fig. 2B), suggesting that C-CAM was either not expressed or expressed in very low levels in these cells (Fig. 2, B and C). In PC-L1 and PC-L2 cells, a single 3.0-kilobase C-CAM1 mRNA was detected. Two PC-CAM transcripts were found in PC-L6 and PC-L9 cells, the 3.0-kilobase-transcript and a slightly larger one (Fig. 2B). This larger transcript may represent a fusion of the C-CAM and neo' genes, because this band was hybridized to both the C-CAM and neo' probes (Fig. 2B). As predicted, the neo' mRNA was expressed in all transfected clones but not in the parental PC-3 cells.
Hybridization with 28S rRNA, which served as an internal control, showed that similar amounts of RNA were loaded in each lane.

Western blot analysis with anti-C-CAM antibody Ab669 revealed similar profiles of C-CAM protein expression in all four C-CAM1 transfectants, and PC-3 and PC-RSN cells were negative for C-CAM expression (Fig. 2C). The C-CAM1 protein expressed from PC-3 transfectants was heterogeneous; the $M_r$ 105,000 form was predominant. The heterogeneity may be due to different degrees of glycosylation since there are 16 potential N-linked glycosylation sites present in the extracellular domain of C-CAM1. More C-CAM1 protein was detected in PC-L1 and PC-L2 cells than in PC-L6 and PC-L9 cells (Fig. 2C), suggesting that C-CAM1 protein levels may be regulated differently in these clones.

Cell Adhesion of Transfected Cells. To test the intercellular adhesion of C-CAM1 expressed on the transfected PC-3 cells, the ability of single cells to form aggregates in suspension was measured. The time course of aggregate formation for PC-3, PC-RSN, PC-L1, PC-L2, PC-L6, and PC-L9 cells, as measured by the decrease in the number of single cells, is shown in Fig. 3. PC-3 and PC-RSN cells, which did not express the C-CAM1 protein, did not aggregate. In contrast, all of the C-CAM1 transfectants formed aggregates. These results indicate that the C-CAM1 expressed in PC-3 cells was able to elicit adhesion. Consistent with their higher level of C-CAM1 protein expression, PC-L1 and PC-L2 cells aggregated slightly more than did PC-L6 and PC-L9 cells.

Growth Characteristics of Transfected Cells. The effect of C-CAM1 on cell growth was also analyzed. The four C-CAM1-transfected clones had growth rates (measured by counting the cells with a hemacytometer) about 50% that of control cells (data not shown). Similar results were obtained by measuring the increase in the number of cells by crystal violet staining (Fig. 4A) and by measuring the rate of DNA synthesis by $[^{3}H]$thymidine incorporation (Fig. 4B). Crystal violet staining showed that PC-RSN cells had a growth rate similar to that of parental PC-3 cells. In contrast, the growth rates of the C-CAM1-transfected cells were about 25% that of the control cells 6 days after plating (Fig. 4A). By day 8, PC-L6 and PC-L9 cells were growing faster than PC-L1 and PC-L2 cells, perhaps because of the lower C-CAM1 levels in PC-L6 and PC-L9 cells. The rate of DNA synthesis, measured by $[^{3}H]$thymidine incorporation, was noticeably lower than control levels in all of the C-CAM1-transfected cells (Fig. 4B). This finding suggests that the growth inhibition of C-CAM1-transfected cells may result from an alteration in the cell cycle.

Suppression of Tumorigenicity of PC-3 Cells by C-CAM1 Expression. Normal cells must attach to a substratum to grow, whereas tumorigenic cells do not and therefore can form colonies on soft agar plates (26). To determine the effect of C-CAM1 expression on soft agar colony formation by PC-3 cells, we examined the anchorage-independent growth of the C-CAM1-transfected clones. These four clones formed significantly fewer colonies in soft agar in three independent experiments (Table 1). In addition, the colonies of PC-L1, PC-L2, PC-L6, and PC-L9 cells were smaller than those of PC-3 and PC-RSN cells (data not shown). These observations suggest that expression of C-CAM1 may affect the tumorigenicity of PC-3 cells.

To test this possibility, we measured the ability of these cells to produce tumors in nude mice in vivo. PC-3 cells have been shown previously to be highly tumorigenic when injected into nude mice (27). To test the transfected cells, cells from each clone were injected s.c. into the flanks of male athymic nude mice, and the incidence of tumor formation and the volumes of the tumors were monitored. As shown in Table 2, 60 days after injection, the tumor incidence was 67–94% for PC-3 cells and PC-RSN cells. In contrast, a much lower tumor incidence was observed in mice injected with C-CAM1-transfected cells. In addition, parental PC-3- and PC-RSN-induced tumors generally arose within 3–4 weeks of injection, whereas injecting the same number of transfected cells into nude mice produced fewer or no tumors within the first 30 days (Table 2). In addition, the tumors produced from transfected cells were much smaller (Fig. 5, A and B). These data indicate that expression of C-CAM1 in PC-3 cells suppressed or delayed tumor growth in vivo.
Taken together, the results of these measurements of tumorigenicity demonstrated that expression of C-CAM1 markedly suppressed the tumorigenicity, as well as the tumor growth, of PC-3 cells. These observations, together with the down-regulation of C-CAM in hepatocellular carcinoma (14, 15), prostate carcinoma, and colorectal carcinoma (16), lead to the conclusion that C-CAM1 has tumor-suppressor activity in epithelial tumors.

Increased Tumorigenicity of NbE Cells by Transfection of Antisense C-CAM1 Plasmid. To further examine the tumor-suppressor activity of C-CAM1 in prostate cancer development, we tested whether down-regulation of C-CAM in a nontumorigenic cell line could lead to tumor formation in nude mice. The NbE cell line, a prostatic epithelial cell line derived from the ventral prostate of a Noble rat (28), was chosen because it expresses detectable levels of C-CAM (Fig. 6C) and does not cause tumor formation when injected into nude mice or into the syngeneic host (28). The antisense C-CAM1 plasmid, constructed by inserting the full-length C-CAM1 cDNA into pRSN in the antisense orientation, was used to reduce C-CAM1 expression.

In the first experiment, NbE cells were transfected with an antisense C-CAM1 vector. Transfected cells were selected by increasing concentrations of G418. After growing in 1 mg/ml G418 for 1 month, transfected cells (antisense 1, 1 x 10^6 cells/site) were injected s.c. into nude mice. Sixty days later, tumors appeared at all the antisense 1 injection sites (Table 3A), but no tumors appeared where parental NbE cells had been injected. The antisense-induced NbE tumors were excised from the mouse and cells were cloned from these tumors. Since antisense-transfected NbE cells are resistant to G418 treatment and the cells derived from the nude mouse are not, the antisense-transfected NbE cells can be specifically selected from total tumor tissues by G418 selection. After G418 selection for 1 month, 6 sublines (named Mac-1 to Mac-6) were isolated from 6 individual tumors. The tumorigenicities of these six cell lines were tested together with several control NbE cell lines, as shown in Table 3.

To confirm the previous finding, the same antisense vector was transfected into NbE cells to generate a second transfected NbE cells (antisense 2). Other control NbE cell lines, including those transfected with sense and vector controls, were also generated and selected under the conditions used in the first experiment. When these cell lines were injected into nude mice, the antisense vector induced tumors at an incidence of 50% appeared 40 days after injection (Table 3), with an average tumor volume similar to that of the previous experiment (Table 3), whereas no tumors developed in mice injected with sense and vector controls (Table 3). Histopathological examination of hematoxylin- and eosin-stained tumor tissues from these antisense 2 tumors revealed that they were anaplastic and poorly differentiated (data not shown). In the same in vivo tumorigenicity study, all six antisense sublines, Mac-1 to Mac-6, showed aggressive tumor formation with almost 100% tumor incidence and palpable tumors 14 days after inoculation. These results indicate that transfection of antisense
C-CAM AS A TUMOR SUPPRESSOR IN PROSTATE CANCER

**DISCUSSION**

Recent studies have shown that the cell cycle can be regulated in a positive (stimulatory) or negative (suppressive) manner. Loss of C-CAM expression has been implicated in the development of various cancers, including prostate cancer. The role of C-CAM as a tumor suppressor has been studied extensively, and its expression levels have been shown to correlate with the tumorigenicity of cells.

**Table 1** Anchorage-independent growth of C-CAM-transfected PC-3 cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
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<tbody>
<tr>
<td>PC-3</td>
<td>ND</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>PC-RSN</td>
<td>100%</td>
<td>100±2</td>
<td>89±3</td>
</tr>
<tr>
<td>PC-L1</td>
<td>52±2</td>
<td>45±1</td>
<td>ND</td>
</tr>
<tr>
<td>PC-L2</td>
<td>36±4</td>
<td>45±1</td>
<td>30±1</td>
</tr>
<tr>
<td>PC-L6</td>
<td>ND</td>
<td>ND</td>
<td>50±3</td>
</tr>
<tr>
<td>PC-L9</td>
<td>ND</td>
<td>ND</td>
<td>60±2</td>
</tr>
</tbody>
</table>

*a* Anchorage-independent growth was measured by triplicate counts of the colonies that had formed by day 21 after plating. The results are expressed as percentages of control (PC-RSN or PC-3) ± SE. 

*b* Exp, experiment; ND, not determined. 

**Table 2** Tumor incidence induced by C-CAM-transfected PC-3 cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Exp 1</th>
<th>Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>12/36 (33%)</td>
<td>24/36 (67%)</td>
</tr>
<tr>
<td>PC-RSN</td>
<td>29/36 (80%)</td>
<td>34/36 (94%)</td>
</tr>
<tr>
<td>PC-L1</td>
<td>2/36 (5%)</td>
<td>12/36 (33%)</td>
</tr>
<tr>
<td>PC-L2</td>
<td>0/36 (0%)</td>
<td>2/36 (5%)</td>
</tr>
<tr>
<td>PC-L6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC-L9</td>
<td>ND</td>
<td>0/18 (0%)</td>
</tr>
</tbody>
</table>

*a* Tumors larger than 10 mm³ were considered to be positive. 

ND, not determined.

The expression of C-CAM has been shown to regulate the cell cycle, and its loss has been associated with increased tumorigenicity. The results indicate that the decreased levels of C-CAM in antisense-transfected cells correlate with their increased tumorigenicity.

**Fig. 4.** Growth rate of C-CAM-transfected PC-3 cells. The in vitro growth rate of C-CAM-transfected PC-3 clones was determined by crystal violet staining (A) and by [³H]thymidine incorporation (B). (A) Five thousand cells were plated on a 24-well plate in the presence of T medium containing 1% FBS. The medium was changed every 4 days, and the number of cells was determined every 48 h by the crystal violet assay. Points, mean; bars, SD. (B) Five thousand cells were plated as above. Fresh medium containing 0.1 µCi of [³H]thymidine was added, and after 2 h, the cells were harvested and the amount of [³H]thymidine incorporated into the cells was determined. Columns, mean; bars, SD.

**Fig. 5.** Change in tumor volume of C-CAM-transfected PC-3 cells in vivo. Each animal was given s.c. injections at six sites. The volume of each tumor was recorded weekly, when tumors became palpable. (A) Experiment 1. (B) Experiment 2. Points, mean; bars, SD.

C-CAM1 into NbE cells changed the cells from nontumorigenic to highly tumorigenic.

Southern analyses were used to determine the clonality of antisense NbE-derived sublines, Mac-1 to Mac-6. Two patterns of integration were observed by hybridizing with either the neo probe (Fig. 6A) or the C-CAM probe (Fig. 6B). Mac-1, Mac-2, Mac-5, and Mac-6 had the same DNA integration pattern, while Mac-3 and Mac-4 had a different pattern. These results indicate that these six sublines originated from two different clones. Western immunoblotting indicated that the levels of C-CAM were significantly (10–100-fold) lower in all 6 sublines than in the parental cells (Fig. 6C). This suggests that the increased tumorigenicity of the antisense C-CAM-transfected cells correlated with their reduced C-CAM levels.
negative regulation of cell growth is often found in malignant cells, which exhibit deranged control of cell proliferation. Accumulating molecular genetic evidence has revealed that loss of negative regulators or increase in positive regulators in normal cells will produce cellular abnormality. Most negative regulators (29, 30), referred to as tumor suppressors, have been found to be involved either in direct control of the cell cycle (e.g., Rb, p53, WT-1) or in the signaling pathway leading to cell growth and differentiation (e.g., NF-1). In addition, recent data suggest that genes related to the maintenance of cell architecture and polarity may also function as tumor suppressors (29, 31, 32).

We observed previously that C-CAM expression in the prostate can regulate cell growth through indirect interactions with other cellular molecules that transduce outside signals into the cells. For example, the integrins, which are Ca2+-dependent CAMs known to interact with a variety of extracellular matrices, have been shown to associate with several intracellular tyrosine kinases (33). On the other hand, CAMs have also been shown to participate in the organization of cell architecture by connecting cytoskeleton molecules through an adherens-junction protein complex. Recent molecular genetic analyses (32) suggest that a potential tumor suppressor gene (NF-2) involved in neurofibromatosis belongs to the family of adherens junction molecules. Also, in the case of E-cadherin, at least three cytoplasmic proteins, α-, β-, and γ-catenin, are associated with the cytoplasmic domain of cadherin (34). These associated proteins, which are part of the adherens junction proteins, play important roles in maintaining cellular architecture (35). Consistent with this observation, recently it was found that α- and β-catenin were also associated with the product of a potential tumor suppressor gene involved in colon carcinogenesis, APC (36, 37). These observations suggest that a cell adhesion molecule can regulate cell growth through indirect interactions with a tumor suppressor.

Expression of C-CAM1 may suppress the tumorigenicity of PC-3 cells by changing the cellular architecture of the tumor cells or by creating a new signaling pathway that interferes with tumor cell

Table 3 Tumor incidence and tumor growth induced by antisense C-CAM1-transfected NbE cells

<table>
<thead>
<tr>
<th>NbE clones</th>
<th>Tumor incidence (%)</th>
<th>Tumor volume (mm³)</th>
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<tbody>
<tr>
<td>Parental</td>
<td>0/6 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>Anti sense 1</td>
<td>6/6 (100%)</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>Mac-1</td>
<td>11/12 (92%)</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Mac-2</td>
<td>11/12 (92%)</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>Mac-3</td>
<td>12/12 (100%)</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>Mac-4</td>
<td>12/12 (100%)</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>Mac-5</td>
<td>12/12 (100%)</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Mac-6</td>
<td>12/12 (100%)</td>
<td>44 ± 6</td>
</tr>
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</table>

"Tumors larger than 10 mm³ were considered to be positive."
growth. The cytoplasmic domain of C-CAM1 contains several potential phosphorylation sites, including one for cAMP-dependent kinase and one for tyrosine kinase. The putative tyrosine phosphorylation sequence is also within the consensus sequence for the antigen receptor homology domain (38) that is postulated to be important for signal transduction from membrane-bound IgM molecules in B cells. These structural features suggest that the cytoplasmic domain of C-CAM1 may be critical for signal transduction and that C-CAM1 may interact with other kinases to elicit a negative signal for cell growth. It is also possible that the cytoplasmic domain of C-CAM1 interacts with cytoskeletal proteins that modulate cellular morphology. Elucidating the proteins C-CAM1 interacts with may reveal how C-CAM1 suppresses tumor formation.

C-CAM1 may also play a role in the control of cell growth in vitro. In this study, the cells expressing C-CAM1 grew one-fourth as fast as cells that did not express C-CAM (Fig. 44). Furthermore, [3H]thymidine incorporation experiments (Fig. 4B) showed a similar pattern of reduction, suggesting that expression of C-CAM1 may be able to slow the transition of the transfected cells from G1 to S phase. The reduced growth potential of the transfected cells was also reflected in the decrease in size of soft agar colonies (data not shown). In addition, the anchorage-independent growth assay also suggested that the transfected cells may become less tumorigenic; these cells formed about one-half as many soft agar as did control cells (Table 1). As a result, we expected that C-CAM1 expression may be able to prolong the tumor latency. Consistently, our in vivo tumorigenicity study showed that elevated C-CAM1 expression reduced the size as well as the number of tumors induced by PC-3 cells in nude mice. These observations together suggest that C-CAM1 may affect not only the tumor growth rate but also other processes involved in tumor formation, such as cell attachment, cellular interaction, and angiogenesis.

In this study, the cells expressing C-CAM1 were able to form aggregates (Fig. 3), indicating that the transfected C-CAM1 gene was functional. As a result, whether adhesion activity is required for the tumor-suppressive action of C-CAM1 is not clear. It is possible that the structural requirement for adhesion activity may be different from that for tumor suppression; adhesion may only require “recognition,” while tumor suppression may require signal transduction in addition to the initial adhesion event. For example, studies by Jaffe et al. (39) of chimeric molecules of N-CAM and L-CAM have shown that the cytoplasmic domains do not affect the adhesion activity but are required for cell sorting and cell patterning. We have previously shown that deletion of the first immunoglobulin domain of C-CAM1 abolishes the adhesion activity (11). It will be interesting to see whether this deletion will also affect the tumor-suppressive activity of C-CAM1.

A common problem associated with the study of tumor suppressors is that it is difficult to obtain transfected clones because expression of a tumor suppressor often results in cell senescence and cell death. Our initial attempts failed to yield a single clone expressing C-CAM1 because the low percentage of C-CAM1-expressing cells among G418-resistant cells prohibited further studies (Fig. 1A). It is possible that in the total G418-resistant cell population, the faster-growing C-CAM1-negative cells outgrew the C-CAM1-positive cells. We eventually took advantage of the fact that C-CAM1 is a cell surface protein and used FACS to enrich the C-CAM1-positive cell population by antibody staining (Fig. 1B). This approach was crucial in obtaining these growth-compromised C-CAM1-expressing cells. An alternative approach to studying the tumor suppressor action of a molecule is to reduce its expression by using an antisense expression vector. We also took this approach by transfecting an antisense C-CAM1 expression vector into a rat prostatic epithelial cell line, NbE, that expresses C-CAM (Fig. 6C). The cell line NbE is derived from the ventral prostate of a Noble rat and is nontumorigenic when injected s.c. into athymic nude mice or a syngeneic host (28). This cell line still retains some normal cell characteristics. For example, the cells form a dome structure (pseudolumen) in the confluent stage; the C-CAM levels were also up-regulated in this dome area (data not shown). Results from Table 3 and Fig. 6 indicate that the six antisense clones (Mac-1 to Mac-6) with reduced C-CAM expression became tumorigenic in nude mice, whereas the parental cells and other control-transfected cells remained nontumorigenic. Data from Southern blot analyses (Fig. 6, A and B) indicate that these clones were derived from two different cells, based on their distinct DNA integration patterns. These results suggest that the increased tumorigenicity of Mac sublines is not due to random insertion from transfection artifact. These results are consistent with the reduced expression of C-CAM in malignant cells seen in human prostate specimens. Therefore, these results further suggest that C-CAM1 plays a tumor suppressor role in prostatic tumorigenesis.

Our previous results indicated constant levels of C-CAM associated with stem/amplifying cells during androgen deprivation. Therefore, we hypothesized that C-CAM may be a growth represser that maintains homeostasis of normal prostate development. In this study, we further demonstrated that increasing C-CAM expression in the highly tumorigenic PC-3 cells resulted in tumor suppression and that reducing C-CAM levels in nontumorigenic NbE cells led to tumor formation. The biochemical and biological properties of NbE cells (28) may resemble those of the potential stem/amplifying cell population described by Isaacs and Coffey (17). For example, NbE cells express cytokeratin, which is often found in stem/amplifying cells. Very interestingly, this cell line is capable of forming a dome and polarizing in semipermeable matrix (data not shown), implying that it retains the potential to differentiate under the influence of the microenvironment. Taken together, these data suggest that C-CAM levels in stem/amplifying cells may play a critical role in determining normal prostate development; therefore, alteration of C-CAM levels may initiate early processes in tumorigenesis.

Since the development of effective local treatment of prostate cancer, prostate cancer mortality has almost always been associated with the appearance of androgen-independent cells at metastatic sites. These androgen-independent cells have been characterized as highly aggressive and poorly differentiated. From this study, we learned that one can control the abnormal phenotype of an androgen-independent cell line, PC-3, by introducing a transmembrane CAM that may interact with the signaling machinery or reorganize cytoskeletal structure. Further understanding of the role of C-CAM1 in prostate cancer progression will certainly have an impact on refining therapeutic strategies.

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